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Accumulation of *Chiro*-inositol and Other Non-structural Carbohydrates in *Limonium* Species in Response to Saline Irrigation Waters

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Abstract. **Two statice cultivars, *Limonium perezii* cv. Blue Seas and *L. sinuatum* cv. American Beauty, were grown in greenhouse sand tanks to determine the effect of salt stress on carbohydrate accumulation and partitioning. For the first experiment, irrigation waters were prepared to simulate typical saline-sodic drainage effluent in the San Joaquin Valley of California with electrical conductivities of 2.5, 7, 11, 15, 20, 25, and 30 dS·m^{-1}. A second experiment compared responses to two types of irrigation waters with salinity levels of 2.5, 6, 8, 10, 12, 16, and 20 dS·m^{-1}: 1) San Joaquin Valley drainage waters, and 2) solutions mimicking concentrations of Colorado River water, a major irrigation water source for southern California. In addition to the presence of *myo*-inositol and three common sugars (fructose, glucose, and sucrose), *chiro*-inositol was for the first time isolated and identified in leaf and root tissues of both *Limonium* species. As salinity increased from 2.5 to 30 dS·m^{-1}, leaf *chiro*-inositol concentration increased from 6.4 to 52.8 and from 2.6 to 72.9 μmol·g^{-1} dry weight for *L. perezii* and *L. sinuatum*, respectively, suggesting that *chiro*-inositol contributes substantially to osmotic adjustment in the stressed plants. Meanwhile, leaf *myo*-inositol concentration remained low in both species and showed little response to salinity. Before salt stress, the seedlings contained little *chiro*-inositol, indicating that salt enhanced *chiro*-inositol synthesis per unit of biomass formation. Significant ($P \leq 0.05$) increasing trends for fructose and glucose and a decreasing trend for sucrose with increasing salinity were observed in the leaves of *L. perezii* but not *L. sinuatum*. As a result, the leaves of *L. perezii* had higher glucose and fructose but lower sucrose levels than that of *L. sinuatum*. However, no significant ($P > 0.05$) salt effect was found on the sum of the three common sugar concentrations in either species. Therefore, the accumulation of *chiro*-inositol resulted in a change in carbon partitioning among the soluble carbohydrates (i.e., the ratio of leaf *chiro*-inositol over a sum of the three common sugars rose from 0.034 to 0.29 dS·m^{-1} and from 0.012 to 0.32 dS·m^{-1} for *L. perezii* and *L. sinuatum*, respectively, as salinity increased from 2.5 to 30 dS·m^{-1}). Salt stress did not affect starch accumulation and caused no carbon reserve deficiency. Furthermore, it was observed that salinity increased *chiro*-inositol phloem transport. The *chiro*-inositol response might be a physiological process for *Limonium* salt adaptation. The types of saline irrigation waters (i.e., sodium sulfate-dominated waters vs. a sodium chloride system) appear to have little effect on carbohydrate accumulation and partitioning in *L. perezii*.**

Improvement of plant salt tolerance relies greatly on understanding how plants deal with salt stress through physiological and biochemical responses. In addition to specific ion toxicity, salinity causes water stress via lowering osmotic potential (ψ_S) in root media. Under salt stress, halophytes may take up and compartmentalize inorganic ions into vacuoles to prevent salt-induced inhibition of enzymatic activities in the cytoplasm (Flowers et al., 1986). High vacuolar ion accumulation lowers cell ψ_S, which facilitates cell water retention and uptake and thus maintains cell turgor. This is balanced by cytoplasmic water potential adjustment through accumulation of large quantities of specific metabolic osmotica or compatible solutes (Glenn et al., 1999). This mechanism for plant adaptation to salinity and survival under high salt stress is well recognized (Popp and Smirnoff, 1995). In addition to lowering ψ_S, compatible solutes can also provide various protective functions to alleviate the dehydration-induced overproduction of toxic oxidative reactive agents and the disruption of membrane and protein structure and function (Bohnert and Shen, 1999). Studies have shown that compatible solute accumulation is correlated with salt tolerance (Popp and Smirnoff, 1995; Subbarao et al., 2001) and appears to be the most effective and fundamental adaptative mechanism for enhancing plant salt tolerance (Hare et al., 1998).

Metabolic osmotica fall into several classes, including nitrogenous compounds (quaternary ammonium compounds, polyamines, and free amino acids) and carbohydrates like sugars (sucrose, glucose, and fructose), polyols or sugar alcohols (mannitol, sorbitol, ononitol, D-pinitol, and inositols), and oligosaccharides (trehalose) (Hare et al., 1998; Williamson et al., 2002). The adaptive value of compatible solutes depends on whether their concentrations increase sufficiently in plant tissues experiencing increasing levels of salt stress (Gorham et al., 1981). With regard to sugar alcohols, D-pinitol, a methylated cyclitol, was found as a responsive metabolite, occurring in a number of halophytes such as mangrove species, *Limonium gmelinii*, and *Mesembryanthemum crystallinum* (Murakeözy et al., 2002; Paul and Cockburn, 1989; Popp,

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1984). Its compatibility has been confirmed, and its cellular location, biosynthetic pathway, and role in plant drought and salt tolerance have been studied in detail (Dittrich and Korak, 1984; Nguyen and Lamant, 1988; Orthen et al., 2000; Paul and Cockburn, 1989). Other cyclitols, such as *chiro*-inositol, have been found in halophytic species of *Aegialitis annulata*, *L. gmelinii*, and *L. latifolium* (Gagneul et al., 2007; Murakeözy et al., 2002; Popp, 1984). However, limited research has been done on their physiological role in salt tolerance, and to our knowledge, no research has even systematically shown whether their accumulation responds to an increasing series of osmotic or salt stresses.

The leadwort family, Plumbaginaceae, includes more than 50 halophytic *Limonium* species, many of which are known to complete their life cycles under hypersaline conditions [i.e., 56 $dS \cdot m^{-1}$ (Aronson, 1989)]. Osmotic adjustment is achieved in the cytoplasm of these species through biosynthesis and accumulation of low-molecular-mass organic solutes. Previous research on their osmotic metabolite accumulation in response to salinity has focused mainly on betaines and other quaternary ammonium compounds (Hanson et al., 1991). Alarcón et al. (1999) speculated that soluble carbohydrates might contribute significantly to osmotic pressure buildup in *L. latifolium*. More recently, the experimental assessment of compatible solute function in *L. latifolium* salt tolerance revealed that the major organic contributors to its osmotic adjustment were *chiro*-inositol and other soluble carbohydrates (Gagneul et al., 2007). Also, D-pinitol was identified in Hungarian inland native *L. gmelinii* as a significant component of the soluble carbohydrate pool, fluctuating with seasonal dynamic salt stress (Murakeözy et al., 2002). With regard to *L. perezii* and *L. sinuatum*, two valuable members in U.S. floral industry, what kinds of sugar or sugar alcohols they accumulate and how their carbohydrate pools respond to salt stress are still open questions. The goal of this study was to determine profiles of sugar and sugar alcohol accumulation in *L. perezii* and *L. sinuatum* grown under a wide range of saline treatments. Starch reserves were also examined to provide more comprehensive information on *Limonium* nonstructural carbohydrate accumulation in response to salinity.

Materials and Methods

Plant materials and growth conditions. The study consisted of two experiments conducted in greenhouse sand tank cultures at the USDA-ARS U.S. Salinity Laboratory, Riverside, CA (lat. 33°58′24″N, long. 117°19′12″W). The tanks measured 1.2 × 0.6 × 0.5 m deep and contained washed sand having an average bulk density of 1.4 $Mg \cdot m^{-3}$. The sand had a volumetric water content at saturation of 0.34 and 0.1 $m^3 \cdot m^{-3}$ after drainage had nearly ceased. Growth conditions and additional experimental details for Expt. 1 are as described in Grieve et al. (2005). Briefly, 15 seedlings of *L. perezii* cv. Blue Seas were transplanted into each of 21 sand tanks on 8 Feb. 2001; 15 seedlings of *L. sinuatum* cv. American Beauty were transplanted into each of a second set of 21 tanks on 16 Mar. 2001. Planting dates were staggered to assure that both species flowered at about the same time. Tanks were irrigated three times daily with a complete nutrient solution. This solution, with an electrical conductivity (EC) of 2.5 $dS \cdot m^{-1}$, served as the control treatment. Salinizing salts were added to the nutrient solutions irrigating *L. perezii* on 15 Feb. and to those irrigating *L. sinuatum* on 7 Apr. Treatments were designed to simulate saline-sodic wastewaters commonly present in the San Joaquin Valley (SJV) of California and from predictions based on appropriate simulations of what the long-term compositions of the water would be upon further concentration by plant-water extraction and evapotranspiration (Suarez and Simunek, 1997). The experimental design was a randomized block with seven salinities (2.5, 7, 11, 15, 20, 25, and 30 $dS \cdot m^{-1}$) (Table 1), two *Limonium* species, and three replications.

Expt. 2 was conducted the next year (2002) in the same greenhouse sand tanks as described above. For this experiment, however, the test crop was *L. perezii* only and seeds were planted directly into the sand tanks on 18 Jan. 2002. After emergence, the plants were thinned to 15 seedlings per tank. The objective of this trial was to compare the response of *L. perezii* to two types of saline irrigation waters differing in ion composition. The composition of solution 1 was the same as used in Expt. 1 [i.e., SJV drainage water composition (sodium sulfate-dominated)]. Solution 2 mimicked saline tailwaters often present in the inland valleys of southern California and essentially represents concentrations of Colorado River (CCR) water (sodium chloride system). ECs of the two irrigation water types were 2.5, 6, 8, 10, 12, 16, and 20 $dS \cdot m^{-1}$ (Table 1). Simulations and predictions of the compositions followed Suarez and Simunek (1997). The salinization treatment was initiated at the same time that seeds were sown into the sand tanks. Growth conditions and additional experimental details are given in Carter et al. (2005). Treatments were replicated three times.

Chemicals. *Chiro*-inositol, *myo*-inositol, D-pinitol, and other chemicals unless mentioned were obtained from Sigma-Aldrich (Milwaukee).

Table 1. Compositions of salinizing salts in irrigation solutions simulating increasing salinities typical of those present in San Joaquin Valley (SJV) drainage waters and in saline tailwaters encountered in the inland valleys of southern California typically representing concentrations of Colorado River (CCR) water.

Salinity type	Total salinity	Ca^{2+}	Mg^{2+}	Na^{+}	SO_4^{2-}	Cl^{-}
	($dS \cdot m^{-1}$)	($mol \cdot m^{-3}$)				
SJV						
	2.5	2.5	1.5	13.8	7.0	7.0
	6.0	6.3	4.9	43.7	21.0	21.1
	7.0	7.8	5.5	50.9	26.0	24.7
	8.0	8.3	6.6	58.2	29.5	28.2
	10.0	10.4	8.3	73.3	37.2	35.5
	11.0	11.8	9.3	82.0	42.0	38.5
	12.0	12.6	10.0	88.5	44.9	42.8
	15.0	13.0	13.9	123.0	58.2	59.6
	16.0	13.4	15.5	137.0	63.9	66.4
	20.0	13.5	20.1	178.0	79.0	86.3
	25.0	13.8	27.9	247.0	104.0	111.0
	30.0	14.0	33.6	298.0	124.0	144.0
CCR						
	2.5	2.8	4.0	14.0	4.0	16.5
	6.0	5.7	9.4	32.3	10.0	42.0
	8.0	7.6	12.7	43.6	13.6	57.2
	10.0	9.4	16.3	55.0	17.3	72.5
	12.0	11.4	19.4	66.9	20.9	87.8
	16.0	15.6	27.5	93.8	29.3	124.0
	20.0	19.0	35.0	121.0	36.8	160.0

Sample preparation. For Expt. 1, samples were taken on 21 Apr. 2001 for *L. perezii* (89 d old), and 8 May 2001 for *L. sinuatum* (53 d old) after both species had been exposed to salt treatments for a period of about 30% of their time-to-maturity days (67 d for *L. perezii*; 31 d for *L. sinuatum*). For Expt. 2, *L. perezii* was sampled 91 d (19 Apr. 2001) after application of salt stress. Two to five plants, depending on plant size, were taken from one tank as one replication. All samples were taken between 1130 and 1230 HR (midday) in the vegetative growth stage. Leaves of the sampled plants were immediately washed using deionized water and blotted dry. Samples were deep-frozen at –80 °C and then freeze-dried at –48 to –50 °C in a Freeze Dry System/Unitrap 10–102 (Virtis, Gardiner, NY) for 72 h. The dried samples were ground in a Wiley mill to pass a 40-mesh (0.635 mm) screen. For seedling nonstructural carbohydrate distribution, samples were taken directly from seedlings grown in vermiculite-filled tray pots and were processed as above.

Soluble carbohydrate assay. Ground dried tissue (0.1 g) was weighed and incubated in 4 mL of 80% ethanol (v:v) in a 80 °C water bath with shaking for 30 min to extract soluble carbohydrates. Ethanol was decanted and the extraction was repeated three more times. A 4- to 6-mL portion of the pooled ethanol extract was dried in a Speed Vac Concentrator (SAVANT, Farmingdale, NY). The dried extracts were resuspended in 1 mL of deionized water, deionized through coupled anion and cation resin columns (AG1-X8/formate and AG50W-X8/H^+; BIO-RAD, Hercules, CA) so that only neutral forms of the soluble carbohydrates were eluted with deionized water. All the eluate was taken to dryness in the Speed Vac Concentrator. The dried samples were redissolved in deionized water, filtered (0.22 μm pore size), and analyzed using a HPLC system with Dionex CarboPac PA1 column (Dionex, Sunnyvale, CA) connected to an ESA Autosampler (model 542; ESA, Bedford, MA). The separated carbohydrates were detected using an ESA Coulochem II Electrochemical Detector and quantified by comparison with known standards.

Isolation and identification of cyclitols. Aliquots of deionized samples and standards were applied to different lanes on Whatman No.1 filter paper sheet (0.23 × 0.58 m). The paper chromatograms were developed in descending mode in a mobile phase of n-propanol:ethyl acetate:water (7:1:2, v/v) for 36 to 38 h for separation. The separated cyclitols were detected using the silver nitrate staining method (Trevelyan et al., 1950) and were identified by comparison with cyclitol standards. For further confirmation of *chiro*-inositol and *myo*-inositol identity, the areas on unstained lanes for the two cyclitols were separately excised and cut into fine strips. The samples were then eluted into 10 mL of 50% ethanol at 55 °C in a shaking water bath. This was repeated two more times, and the pooled eluate was filtered (0.45 μm pore size) and dried in a Speed Vac Concentrator. The resulting pellets were redissolved in deuterium oxide, filtered through a 0.22-μm filter, and identified using an INOVA300 nuclear magnetic resonance (NMR) spectrometer (Varian, Palo Alto, CA) at 500 MHz (^{1}H) according to Ichimura et al. (1998).

Starch assay. The assay was based on procedures used by Liu et al. (1999). The extracted residues were oven dried at 55 °C, suspended in 2.0 mL of 2N KOH, and boiled for 1 h to gelatinize the starch. After cooling, the sample was mixed with 2.0 mL of 2N acetic acid to adjust its pH to 4.5. The starch was then hydrolyzed to glucose using amyloglucosidase (Fluka, Ronkonkoma, NY; 93 units per sample). The resulting glucose was detected with a glucose kit (HK20; Sigma-Aldrich) using a scanning spectrophotometer (PowerWave$_x$ Select Microplate; Bio-Tek, Winooski, VT) at 340 nm, and was quantified by comparison with a known glucose standard.

Phloem sap collection and analysis. The experimental protocol for phloem exudate collection was essentially that used by Liu et al. (2002). Fully expanded leaves were excised midday in full sun, and their petiole ends were immersed in deionized water and transferred to the laboratory from the greenhouse. The excised petiole ends were then immersed in 20 mM Na_2EDTA, pH 7.0, in a petri dish and were recut at an angle leaving a stub of a standard 2 to 3 mm length attached to the leaf base. The petiole stub was then immersed in 20 mM Na_2EDTA, pH 7.0, in a 1.5-mL microcentrifuge tube. Phloem exudation was allowed to proceed under ambient room light and temperature conditions and the exudates were deionized and analyzed for carbohydrates as outlined above.

Statistical analysis. The significance of salt effects on sugar, polyol, and starch concentrations and of their differences between species or between salt compositions were analyzed at $P \leq 0.05$ using GLM and TTEST procedures, respectively, in SAS (version 8.2; SAS Institute, Cary, NC).

Results

Occurrence of *chiro*-inositol. Paper chromatography and HPLC analyses showed that *L. perezii* and *L. sinuatum* possibly contained not only *myo*-inositol but also *chiro*-inositol. NMR ^{1}H spectra for the two individual isolated polyols had all the peaks with the same chemical shift sets as for *myo*-inositol and *chiro*-inositol standards, respectively. This confirmed that not only *myo*-inositol but also the rare cyclitol *chiro*-inositol (Fig. 1) occurred in both species. Because we did not examine the stereocenter of the isolated *chiro*-inositol, D-*chiro*-inositol and L-*chiro*-inositol have the same HPLC retention time, paper chromatography mobility, and NMR spectrum (data not shown), we are uncertain at this time whether D- or L- or both types of *chiro*-inositol occurred in the *Limonium* species examined. Here, it is simply reported as *chiro*-inositol. Murakeözy et al. (2002) reported that *L. gmelinii* ssp. *hungarica* accumulated pinitol, a common stress-inducible cyclitol, as one of its prominent soluble carbohydrates. We particularly examined whether these two *Limonium* species accumulated pinitol using HPLC, paper chromatography, and NMR. However, pinitol was not detected in the shoots of *L. perezii* or *L. sinuatum* under salt or nonsalt stress conditions.

Distribution of soluble carbohydrates and starch in seedlings. In addition to *chiro*-inositol and *myo*-inositol, fructose, glucose, and sucrose were also detected in *L. perezii* and *L. sinuatum*. These three common sugars accounted for more than 92% of the total soluble carbohydrates (w/w) in 29-d-old seedlings before salt treatment (Table 2). *Chiro*-inositol and *myo*-inositol accounted for the remaining 8%. Only small amounts of these two inositols were found in leaves and roots and their concentrations did not exceed 2.7 and 15.7 $\mu mol \cdot g^{-1}$ dry weight for *chiro*-inositol and *myo*-inositol, respectively (Table 2). The other major carbohydrate reserve form, starch, accumulated mainly in the leaves, with concentrations of 166 and 204 $mg \cdot g^{-1}$ dry weight for *L. perezii* and *L. sinuatum*, respectively. In the roots, starch concentration was low, with

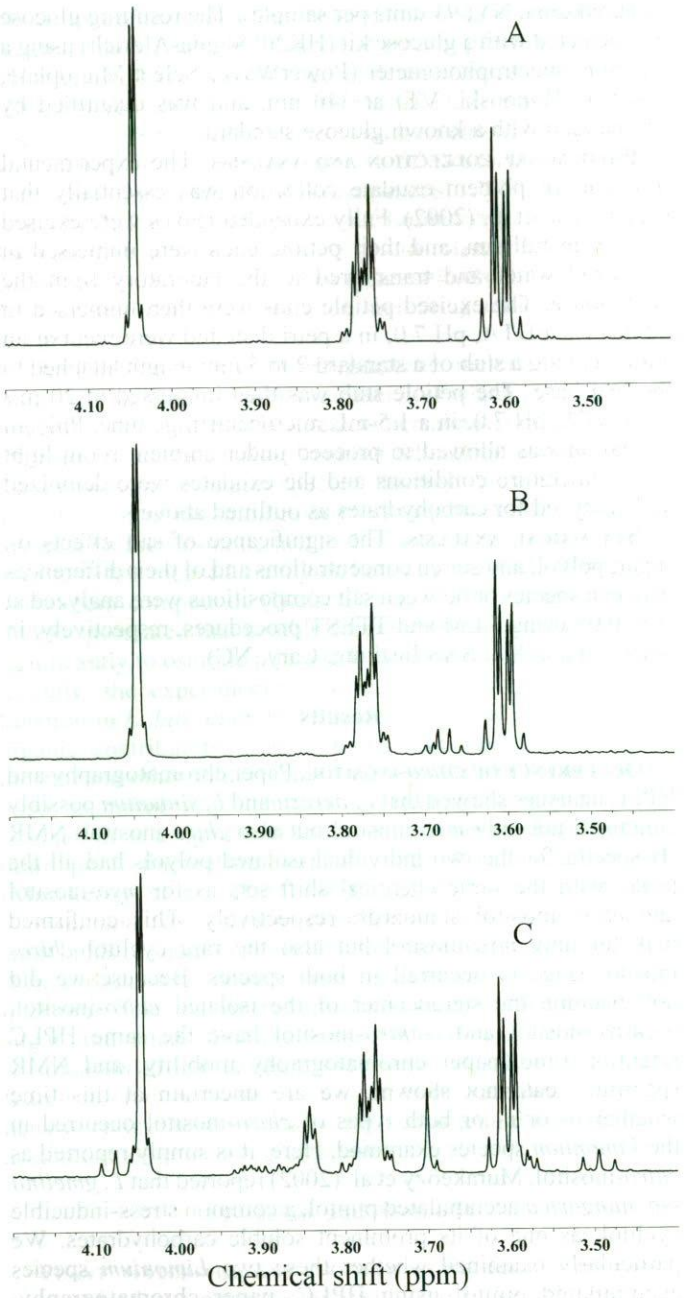

Fig. 1. Proton nuclear magnetic resonance (^{1}H NMR) spectra of *chiro*-inositol, standard (**A**) and isolated from *Limonium perezii* (**B**), and *Limonium sinuatum* (**C**) leaves. Additional peaks in the isolated samples are sample noise peaks.

the values of 26.5 and 25.8 mg·g^{-1} dry weight for *L. perezii* and *L. sinuatum*, respectively (Table 2).

Response of carbohydrate accumulation to salinity. Soluble carbohydrate and starch concentrations in the leaves of salt-stressed plants were determined (Fig. 2). *Chiro*-inositol and *myo*-inositol concentrations were low in nonsalt-stressed plants, with the lowest values being 6.4 and 17.1 (*L. perezii*), and 2.6 and 10.4 (*L. sinuatum*) μmol·g^{-1} dry weight, respectively (Fig. 2, A and B). Increased leaf *chiro*-inositol accumulation correlated with increased salinity for both *Limonium* species. As salinity increased to 30 dS·m^{-1}, leaf *chiro*-inositol concentration increased significantly ($P \leq 0.05$), reaching 52.8 and 72.9 μmol·g^{-1} dry weight for *L. perezii* and *L. sinuatum*, respectively (Fig. 2A). Compared with concentrations in unstressed leaves, these values were 8.3- and 28.0-fold higher for *L. perezii* and *L. sinuatum*, respectively. Under salt stress conditions, *chiro*-inositol became the predominant carbohydrate. *Myo*-inositol concentration in each species, however, was not significantly ($P > 0.05$) affected as salinity increased from 2.5 to 30 dS·m^{-1} (Fig. 2B). Overall, no significant ($P > 0.05$) difference in leaf cyclitol accumulation between the two *Limonium* species was found in response to increasing salinity (Fig. 2, A and B).

Significant ($P \leq 0.05$) differences of leaf hexose (glucose and fructose) concentrations for both species were found at different salinity levels (Fig. 2, C and D). Specifically, leaf hexose concentrations in *L. perezii* increased as salinity increased and then reached a plateau when salinity reached 15 and 10 dS·m^{-1} for fructose and glucose, respectively. In contrast, hexoses were relatively constant in *L. sinuatum* under salt stress conditions. Sucrose concentration decreased significantly ($P \leq 0.05$) from 57.0 to 14.7 μmol·g^{-1} dry weight in *L. perezii* but showed no significant ($P > 0.05$) change in *L. sinuatum* as salinity increased from 2.5 to 30 dS·m^{-1} (Fig. 2E). As a result, leaves of *L. sinuatum* had higher sucrose concentration than those of *L. perezii* under salt stress. However, no significant ($P > 0.05$) difference in salt effect was found on the sum of the three common sugar concentrations between species. Overall, there was a significant ($P \leq 0.05$) salt effect on leaf total soluble carbohydrate concentration for both species. In *L. sinuatum*, leaf total soluble carbohydrate concentration increased from 178.7 to 253.7 μmol·g^{-1} dry weight as salinity increased from 2.5 to 30 dS·m^{-1} (Fig. 2F). In *L. perezii*, leaf total soluble carbohydrate level increased with increasing salt stress from 151.2 to reach a plateau around 251.3 μmol·g^{-1} dry weight at a salinity of 15 dS·m^{-1} (Fig. 2F). No significant ($P > 0.05$) salt effect was found on leaf starch concentration in either *Limonium* species (Fig. 2G).

While salinity did not alter total carbohydrate content, it did alter carbon partitioning between soluble carbohydrates, as shown by the significant ($P \leq 0.05$) increase in the ratio of *chiro*-inositol over the sum of fructose, glucose, and sucrose from 0.034 to 0.29 (*L. perezii*) and from 0.012 to 0.32 (*L. sinuatum*) as salinity increased from its control level to 30 dS·m^{-1} (Fig. 2H), a 8.5- and 26.7-fold increase for *L. perezii* and *L. sinuatum*, respectively.

Expt. 2 was designed to compare sugar, polyol, and starch accumulation in *L. perezii* grown under conditions mimicking SJV drainage water salt composition versus CCR drainage water salt composition. Essentially, leaf *chiro*-inositol, *myo*-inositol, fructose, glucose, sucrose, and starch concentrations as well as the ratio of *chiro*-inositol content over the sum of fructose, glucose, and sucrose in response to either type of salinity (Fig. 3) echoed what were found in Expt. 1 with *L. perezii* grown in a salinity range from 2.5 to 20 dS·m^{-1} (Fig. 2). Overall, the relatively higher SO_4^{2-} and lower Cl^- constituents found in SJV drainage water compared with CCR waters (Table 1) resulted in no significant ($P > 0.05$) difference in total soluble carbohydrate or starch concentrations in *L. perezii* leaves under the different salinities (Fig. 3, A–G). The same was true for the carbon partitioning, the ratio of *chiro*-inositol over the sum of fructose, glucose, and sucrose (Fig. 3H).

Phloem transportation of *chiro*-inositol. Detached source leaves of *L. perezii* exuded sugars and sugar alcohols

Table 2. Distribution of soluble carbohydrates and starch in the leaves and roots of *Limonium* perezii and *Limonium sinuatum* of 29-d-old seedlings (with a dry shoot biomass of 0.024 g and 0.38 g per plant for *L. perezii* and *L. sinuatum*, respectively) grown under nonsalt-stress condition.

	Chiro-inositol	*Myo*-inositol	Fructose	Glucose	Sucrose	Total soluble carbohydrates	Starch
	[mean ± SE (μmol·g^{-1} DW)]^z						(mg·g^{-1} DW)
L. perezii							
Leaf	2.7 ± 0.7	15.7 ± 0.5	44.9 ± 0.9	42.2 ± 1.9	82.7 ± 5.4	188 ± 7.8	166 ± 3.9
Root	1.5 ± 0.1	6.8 ± 0.1	51.9 ± 1.0	32.7 ± 0.5	120 ± 1.9	213 ± 0.2	26.5 ± 2.0
L. sinuatum							
Leaf	2.3 ± 0.2	15.4 ± 1.2	42.1 ± 8.1	37.9 ± 5.8	95.6 ± 11.3	193 ± 26.0	204 ± 16.7
Root	1.8 ± 0.1	7.7 ± 0.8	49.6 ± 4.2	37.5 ± 3.5	159 ± 18.3	256 ± 26.9	25.8 ± 1.7

^{z}n = 3 for leaves and n = 2 for roots (a pooled sample of 12–40 plants was for one replication).

from their petioles into Na-EDTA solutions (Fig. 4). *Chiro*-inositol and *myo*-inositol were phloem-transportable, but apparently much more *chiro*-inositol was exuded than *myo*-inositol (Fig. 4, A and B). Sucrose and *chiro*-inositol were the predominant carbohydrates in phloem-exuded sap. Salt-stressed leaves exuded significantly ($P \leq 0.05$) more *chiro*-inositol than did nonsalt-stressed leaves throughout the exudation period (Fig. 4A), which made *chiro*-inositol the predominant soluble carbohydrate on a molar basis (Fig. 4, A and C). The amounts of exuded *chiro*-inositol reached 0.023 and 0.078 μmol·g^{-1} dry weight after 12 h exudation for nonsalt-stressed and salt-stressed leaves, respectively. However, differences in the amount of *myo*-inositol or sucrose exuded between nonsalt- and salt-stressed leaves were not significant ($P > 0.05$) over the exudation period (Fig. 4, B and C). In late exudation period, the amount of sucrose exuded by non-stressed leaves appeared higher than that of stressed leaves (Fig. 4C).

Discussion

We have here demonstrated the presence of *chiro*-inositol in *L. perezii* and *L. sinuatum*. Recently, *chiro*-inositol was detected and quantified in *L. latifolium* (Gagneul et al., 2007).

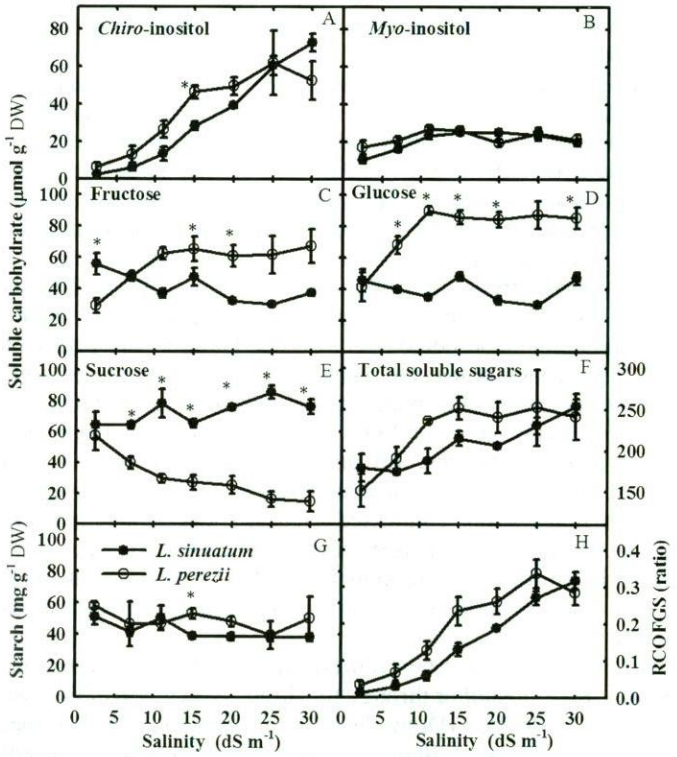

Fig. 2. *Limonium perezii* and *Limonium sinuatum* leaf soluble carbohydrate (**A**–**F**) and starch (**G**) concentrations, and the ratio of *chiro*-inositol over the sum of fructose + glucose + sucrose (RCOFGS) (**H**) in response to salinity (San Joaquin Valley drainage water salt composition). Total soluble carbohydrates are the sum of all the detected sugars and sugar alcohols. Vertical bars represent ± SE, n = 3 (tanks). Significant difference ($P \leq 0.05$) between the two species is marked with an asterisk at any given salinity level.

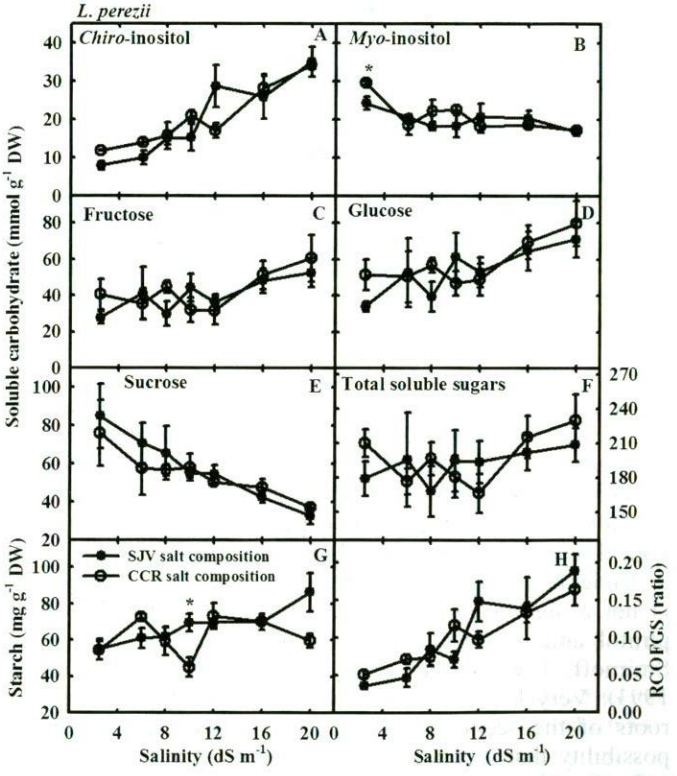

Fig. 3. Comparison of responses of *Limonium perezii* leaf soluble carbohydrates (**A**–**F**) and starch (**G**) concentrations, and ratio of *chiro*-inositol over the sum of fructose + glucose + sucrose (RCOFGS) (**H**) to two salt compositions, San Joaquin Valley (SJV) drainage waters versus saline tailwaters encountered in the inland valleys of southern California typically representing concentrations of Colorado River (CCR) water. Total soluble carbohydrates are the sum of the all detected sugars and sugar alcohols. Vertical bars represent ± SE, n = 3 (tanks). Significant difference ($P \leq 0.05$) between the two salt compositions is marked with an asterisk at any given salinity level.

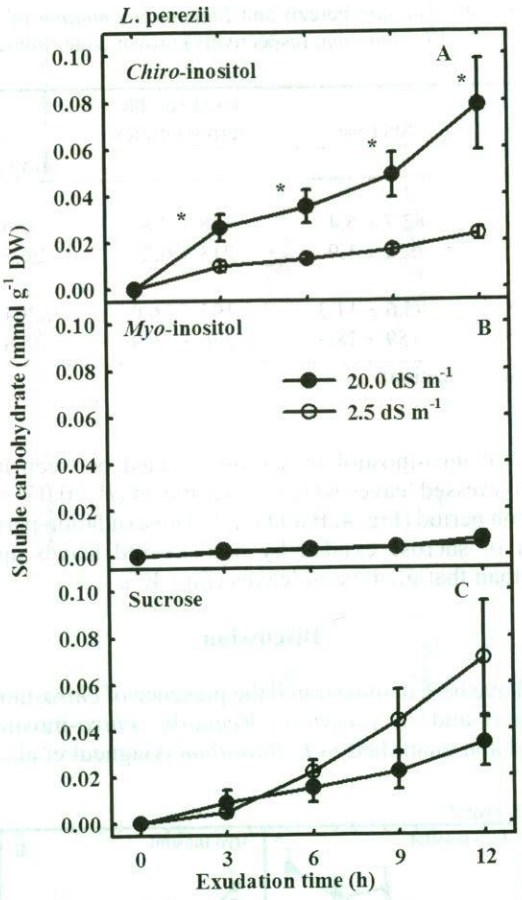

Fig. 4. Cumulative phloem exudation of cyclitols (**A** and **B**) and sucrose (**C**) from fully expanded leaves of *Limonium perezii* under salt (20.0 dS·m^{-1}) and nonsalt stress (2.5 dS·m^{-1}). The two sources of saline water, San Joaquin Valley drainage waters versus saline tailwaters encountered in the southern inland valleys of California typically representing concentrations of Colorado River water, had the same effect on the carbohydrate exudation, and thus the data were pooled. Vertical bars represent ± SE, n = 8 (leaves). Significant salt effect at each exudation period ($P \leq 0.05$) is marked with an asterisk at any given salinity level.

Murakeözy et al. (2002) reported that *L. gmelinii* possibly contained *chiro*-inositol, but gave no quantitative information for this cyclitol. Instead, they confirmed pinitol occurrence and quantified its level in *L. gmelinii*. This suggests that specific cyclitol accumulation may be a species-characteristic physiological feature for halophytic *Limonium* species.

Under nonsalt-stressed conditions, *chiro*-inositol apparently is not a significant metabolite, like the compatible solutes pinitol and betaine (Paul and Cockburn, 1989; Popp and Smirnoff, 1995; Orthen et al., 2000; Rhodes and Hanson, 1993). Very low *chiro*-inositol concentrations in leaves and roots of the seedlings before salt treatment precluded the possibility that a concentrating effect caused by growth rate reduction due to salt stress (Grieve et al., 2005) might be responsible for the high measured *chiro*-inositol level. Instead, the increase in *chiro*-inositol concentration with increasing salinity was an active response through its enhanced synthesis in response to salt stress, which resulted in more carbon allocated to *chiro*-inositol per unit of biomass formation.

Leaves of the both salt-stressed *Limonium* species accumulated amounts of salts (Grieve et al., 2005) that would be toxic to cytoplasmic function if they were not restricted to the vacuoles. The observed increase in *chiro*-inositol accumulation might counteract a high vacuolar ion accumulation by increasing cytoplasmic ψ_S. To appropriately contribute to osmotic adjustment, osmolytes should be stress-inducible and probably in a range between 40 and 400 μmol·g^{-1} (Subbarao et al., 2001). Leaf *chiro*-inositol concentration in both *Limonium* species was 40 μmol·g^{-1} or higher under moderate (15–20 dS·m^{-1}) or higher salt stress, thus contributing significantly to osmotic adjustment. Evidence of cytoplasmic localization of *chiro*-inositol would further verify this function, although its location might be similar to that of pinitol, which was found in cytoplasm but not in vacuoles (Paul and Cockburn, 1989). Fructose, glucose, and sucrose are metabolically labile compounds whose function as compatible cytosolutes is debatable (Gorham et al., 1981). Some experiments on the impact of these solutes on enzymatic activities showed that, in fact, they might be incompatible and more often found in vacuoles (Rozema et al., 1978; Wagner, 1979). *Chiro*-inositol, on the other hand, is quite inert (Popp and Smirnoff, 1995), thus supporting the notion (Gorham et al., 1981) that polyols have little effect on enzyme activities and are more compatible than other solutes. The increase in *chiro*-inositol accumulation observed here mainly accounted for the altered carbon partitioning between *chiro*-inositol and the three common sugars under salt stress, implying that the cells might require more *chiro*-inositol rather than the common sugars to deal with salinity increase. In addition, *myo*-inositol was present in only small amounts and showed little response to salinity. Thus, *chiro*-inositol appears more significant than the other carbohydrates in playing an adaptive role for *Limonium* species salt tolerance.

The observed levels of *chiro*-inositol in *L. perezii* and *L. sinuatum* were similar to levels of D-pinitol in *L. gmelinii* (Murakeözy et al., 2002) and might function similarly in response to salt stress. Under salt stress, structures and functions of membranes and enzymes are subjected to salt damage, and toxic radical oxygen species production may be enhanced to levels causing leaf necrosis (Bohnert and Shen, 1999; Hernández et al., 2001; Mittova et al., 2003). Polyols have water-like hydrogen-hydroxyl groups and can contribute to maintaining an ordered hydration shell around the surface of proteins or membranes and stabilizing protein structures, thus preventing metabolic inactivation under low ψ_S (Timasheff, 1993; Williamson et al., 2002). In addition, polyols have been reported to be effective scavengers of radical oxygen species (Orthen et al., 1994). *L. perezii* and *L. sinuatum* survived all salt treatments and completed their life cycles without any visual symptoms of stress (Grieve et al., 2005). The increased *chiro*-inositol accumulation that paralleled increased salt stress might then be involved in protecting plants from salt-stress damages.

Chiro-inositol can be synthesized by a one-step isomerization of *myo*-inositol or through the direct cyclization of glucose by a cyclo-aldolase (Drew, 1984; Taguchi et al., 1997). Either pathway is simpler than the pathway leading to D-pinitol formation, which occurs through methylation of *myo*-inositol to produce ononitol and the subsequent isomerization of ononitol to pinitol (Dittrich and Korak, 1984). The production of methyl groups used in pinitol synthesis involves photorespiration (Hare et al., 1998), which leads to H_2O_2 production and thus increases the potential for oxidative damage to the cell. We

speculate that the accumulation of *chiro*-inositol instead of D-pinitol might reduce H_2O_2 production, thus favoring the adaptation of these plants to salt stress.

As had been reported for *L. gmelinii* (Murakeözy et al., 2002), we found that *L. perezii* and *L. sinuatum* have high levels of soluble carbohydrate. Although also found in high amounts, nitrogenous compounds found in *L. latifolium* could not by themselves fully account for the metabolite contribution to leaf cell osmotic adjustment (Gagneul et al., 2007). It appears rather that halophytic *Limonium* species accumulate nitrogenous compounds and soluble carbohydrates in high amounts. Here, we found that overall carbohydrate reserves (total soluble sugars, polyols, and starch) in *L. perezii* and *L. sinuatum* appeared to be adequate even though more *chiro*-inositol was synthesized under salt stress. A decrease in sucrose concentration accompanied by an increase in fructose and glucose concentrations was observed in *L. perezii* but not in *L. sinuatum*. *L. sinuatum* is more salt tolerant than *L. perezii* (Grieve et al., 2005). Whether the differential response of the three individual common sugars to salinity was related to differences in salt tolerance between the two *Limonium* species is still to be resolved.

Treatments using sulfate-dominated SJV drainage waters resulted in relatively higher sulfur and sodium but less calcium and chloride accumulation in the leaves than did treatments based on sodium chloride dominated CCR salts (Carter et al., 2005). Differences in composition of the irrigation waters and the resulting differences in specific ion accumulation, however, had little effect on plant carbohydrate accumulation.

Because *chiro*-inositol and *myo*-inositol are phloem-transportable, these polyols are readily translocated from leaves to roots, which links leaf cyclitol pool to roots. For halophytes, this cyclitol phloem flux appears particularly important. In *M. crystallinum*, stress-inducible ononitol and its precursor, *myo*-inositol, act as a leaf-to-root signal. This facilitates sodium uptake and translocation through the xylem to leaves, leading to a transition from a nontolerant to a salinity-tolerant state (Nelson et al., 1999). *Chiro*-inositol phloem-transportation was observed here to be enhanced by salt stress, suggesting a similar correlation between higher salt uptake and higher amounts of cyclitol translocated to roots in *Limonium* species. More evidence on the role of *chiro*-inositol in regulation of salt accumulation is needed for a further understanding of salt tolerance of *Limonium* species.

Chiro-inositol accumulation in response to salinity appears an important feature of *Limonium* salt stress tolerance. Almost all of our modern crops are derived from glycophytes and thus lack a genetic basis for salt tolerance (Glenn et al., 1999). Targeting production of low-molecular-weight solutes is one of the primary strategies currently used for improving crop salt tolerance through breeding or by molecular transformation (Bohnert and Shen, 1999; Nuccio et al., 1999). Because the biosynthesis of *chiro*-inositol needs just one step from *myo*-inositol or glucose, this potentially makes it a better candidate for bioengineering salt tolerance than pinitol, whose synthesis requires relatively one more step. Future progress in characterizing *chiro*-inositol biosynthesis in the *Limonium* species may provide a basis for its bioengineering transfer, which in turn, may provide valuable information on the physiological function of *chiro*-inositol accumulation in plant adaptation to salt stress.

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